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Reciprocal regulation of mir-10b, aurora-a, p53, and e-cadherin in cisplatin resistance and cell invasion of lung cancer

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Abstract

Lung cancer remains a leading cause of cancer-related deaths in developed nations, including Taiwan, mainly due to drug resistance or malignant conditions such as metastasis. Abnormal expression levels of oncogenes or tumor suppressors are recognized as inducing changes and malignancy in various cancers, including lung cancer. This report illustrates that mir-10b, Aurora-A, N-cadherin, and vimentin expression levels are elevated. At the same time, p53 and E-cadherin are reduced in A549cisR clones compared to parental cells, indicating a possible regulatory network among these molecules in malignant neoplasms. A functional assay demonstrated that the reduction of mir-10b or Aurora-A lessened both the resistance to cisplatin and cell motility of A549cisR cells, accompanied by a decrease in vimentin and N-cadherin, while an increase in p53 and E-cadherin. The co-expression of mir-10b agomir restores the drug-resistant and invasive motility of Aurora-A-knockdown A549cisR cells. Besides, ectopic expression of the active form of Aurora-A also increases mir-10b, N-cadherin, and vimentin expressions while decreasing E-cadherin and p53 levels, thus restoring cisplatin resistance and cell motility in mir-10b-knockdown A549cisR cells. Interestingly. Ectopic expression of E-cadherin reduced both motility and resistance to chemotherapeutic drugs, accompanied by altering Aurora-A, p53, and mir-10b levels in A549cisR cells. Subsequent investigations revealed that mir-10b secretion increased in A549cisR cells. Parental A549 cells cultured with a conditioned medium of A549cisR cells showed significantly reduced endogenous p53 expression, while inducing Aurora-A expression and increased viability after cisplatin treatment. Transfection with mir-10b antagomir reversed the expressions of Aurora-A, N-cadherin, vimentin, p53 and E-cadherin and the effects in parental A549 cells cultured in a conditioned medium. These findings suggest that the mir-10b-Aurora-A-E-cadherin pathway is crucial in orchestrating various malignancies, such as invasive motility and drug resistance, offering a possible malignancy-priming route in lung tumor cells with regular cisplatin treatment.

Keywords: Aurora-A, p53, mir-10b, Cadherins, Malignancy.

1. Introduction

Lung cancer ranks as the leading cause of cancer-related deaths in developed nations worldwide. According to global estimates, nearly 1.83 million new cases of lung cancer were diagnosed, accounting for 13.0% of all cancer cases, while about 1.60 million deaths occurred, representing 19.4% of all cancer deaths [1, 2]. In Taiwan, lung cancer has consistently been one of the two leading diagnosed cancers and remains the predominant cause of cancer deaths[3]. Lung cancer is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLCs are further classified into adenocarcinomas, squamous cell carcinomas (SCC), and large cell carcinomas (LCCs) [4]. Approximately 85% of lung cancers are recognized as non-small cell lung cancer, with around 75% presenting as metastatic or advanced upon diagnosis, for which no curative treatment exists. Most patients are diagnosed at advanced stages; it is considered a terminal disease with a 5-year survival rate of less than 20% [5]. These findings indicate the importance of researching novel signaling modules related to drug resistance and/or cell-invasive motility.

Chemotherapy is frequently used to treat cancer patients and is advised in various situations, such as before surgery, after surgery, for locally advanced diseases, and metastatic dissemination. Among chemotherapeutic drugs, cisplatin (CDDP) is considered one of the most potent anticancer drugs, showing substantial clinical effectiveness against a range of solid tumors [6]. For approximately 85-90% of patients with NSCLC who do not have drug-targeted driver mutations, platinum-based chemotherapy continues to be a standard treatment method [7]. Furthermore, CDDP is the more effective platinum agent for patients with advanced NSCLC and those with early-stage disease who

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need induction or adjuvant therapy [7, 8]. Clinically, CDDP-treated patients typically exhibit high response rates. However, in many cases, CDDP-exposed tumor cells can initiate adaptation or resistance mechanisms. As a result, a significant portion of CDDP-treated patients experience treatment failures and tumor recurrence [9, 10]. Furthermore, recurring tumors typically display aggressive motility and metastatic traits [11-13]. Recently, it has been found that genes related to motility are often upregulated in cisplatin-resistant NSCLC cells [14-16]. Furthermore, some studies have noted that these two malignant capabilities of cancer cells may be linked; for example, the transition from an epithelial to a mesenchymal state, an essential step for motility, was found in cisplatin-resistant lung cancer cells through an Akt/β-catenin/snail-dependent pathway [14]. In addition to lung cancer, similar relationships between motility and drug resistance have also been observed in other cancers, such as pancreatic and breast cancer [17, 18], opening avenues for novel therapeutic approaches that target multiple malignancies simultaneously in lung tumors.

Our previous findings have shown that Aurora-A overexpression contributes to drug resistance in lung cancer cells [19]. Our other studies have shown that the tumor suppressor p53 regulates Aurora-A, and the absence of p53 increases the expression of cancerous Aurora-A and is associated with a poor prognosis in patients with lung cancer [20, 21]. Furthermore, we found that the oncogenic mir-10b, known as the molecular driver gene, inhibits the expression of p53 and contributes to resistance to cisplatin [16], suggesting a potential pathway composed of mir-10b, p53, and Aurora-A to modulate motility in drug-resistant tumor cells. Besides, traditional investigations of drug resistance of cancer cells were often through the short-term treatment of cells with a relatively high dose of chemotherapeutic agents, which are usually impossible to apply in clinical treatments due to their high toxicity. Furthermore, various possible effects of long-term, low-dose, and intermittent cisplatin treatment on cancer cells have not yet been clarified. In this study, we have generated cisplatin-adaptive lung tumor cells, and the following researchers discovered that the mir-10b-p53/Aurora-A-cadherin pathway coordinates the resistance to cisplatin and mobility in cisplatin-adaptive lung cancer cells.

2. Materials and methods

2.1. Cell lines, culture conditions, and cisplatin training

The human lung cancer cell line A549 was maintained in RPMI 1640 medium (Gibco/BRL, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Utah, USA), two mM glutamine and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin), at 37 °C in a humidified atmosphere of 5% CO2. To establish cisplatin-treated A549 clones, we periodically exposed A549 cells to cisplatin (treated once for two days and rested for another two days) stepwise from 0.5 to 4 μM by increasing 0.5 μM every two weeks. The resulting descendants of cisplatin-treating A549 cells were then subjected to an indicated viability assay. Cisplatin (Cat NO. 232120) and paclitaxel (Cat NO. 580555) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell proliferation assay

Cell viability was determined by an MTT assay. Briefly, cells were seeded in a 96-well plate at a density of 5×10^3/well; 24 h later, cells were exposed to different concentrations of cisplatin for 48 h. The medium was then replaced with a serum-free culture medium containing 20 μl of MTT (5 mg/ml), followed by incubation for another 2 h at 37 °C. The reaction medium was discarded, washed with 1XPBS three times, and 200 μl DMSO was added to cells in the dark for 5 minutes. The absorbance at 570 nm was measured by a microplate reader (CLARIOstar, BMG Labtech, Offenburg, Germany). The data presented represent at least three separate repeated experiments.

2.3. Cell lysis and western blotting

The cell lysis protocol was performed as previously described [20]; briefly, cells were lysed in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, one mM EGTA, one mM EDTA, 0.5 mM dithiothreitol, 1% NP-40, 0.1% deoxycholate), and protein concentration was determined using the Bradford method. Equal amounts of sample lysates were subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to a PVDF membrane (Millipore, MA, USA). The membrane was blocked with 4% non-fat milk in TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated overnight at 4 °C with specific primary antibodies. Subsequently, the membrane was washed with 1xTBST buffer and incubated with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG). Signal detection was performed using enhanced chemiluminescence kits (Amersham; ECL kit). All western blot exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by the AlphaImager HP densitometer (Proteinsimple, CA, USA).

2.4. Wound-healing assay

Cells were seeded in a 6-well plate and incubated until they reached 90% confluency. A 200-μl pipette tip was used to create a wound, and cells were washed with 1xPBS twice and then replaced with serum-free cultured medium. The cells were treated for 20 h, and the migrating cells from the leading edge were photographed at 0 and 20 h. Gap area coverage is calculated as a percentage of the initial gap closure via ImageJ software.

2.5. Migration assay

For the *in vitro* migration assay, cells were trypsinized, and $2x10^4$ cells were seeded in the upper chamber of the Transwell membrane (pore size: 8 μm; EMD Millipore) for 16 h. The medium was subsequently aspirated, and the filters were washed twice with PBS prior to fixation with methanol for 15 min. Cells were then stained with Giemsa solution to quantify the migrated cells. The migrated cells from the A549cisR cells were normalized against the parent cells as relative fold-change.

2.6. Matrigel invasion assay

The cell invasion assay was performed in a 24-well Transwell unit (8-μm pore size) with 1 mg/ml Matrigel matrix coated on the upper panel as described [22]. Parental or treated cells were placed in the upper compartment of the invasion chamber coated with Matrigel in the presence of 1% serum. A conditioned medium with 10% serum (500 μl) was added to the lower compartment of

the invasion chamber. After incubation at 37 °C for 24 h, cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. The cells in random fields were then counted by light microscopy.

2.7. RNA extraction and reverse transcription-quantitative (RT-qPCR) of miR-10b

The miRNAs of the indicated cells were extracted using the mirVANA® miRNA isolation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions as previously described [23]. 5 µl of total miRNA were used in the reverse transcription reaction with the miRNA reverse transcription kit (Thermo Fisher Scientific, Inc.), using a 5X miR-10b probe. RT-qPCR was performed with the TaqMan PCR master mix kit (Thermo Fisher Scientific, Inc.), using a 20X miR-10b probe. The signals were read using an ABI PRISM 7900 sequence detection system. Reverse transcription and amplification of RNU6B were used as internal controls.

2.8. Cellular transfection

Human mir-10b-agomir and antagomir were purchased or purchased from Thermo Fisher Scientific (MA, USA). Aurora-A siRNA or control siRNA was purchased from MDBio (MDBio, Taiwan). Transfections were performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 18-24 h. The parental or transfected cells were subsequently applied in sequential experiments.

2.9. Statistical analysis

Statistical analysis between groups was performed using an unpaired Student's t-test with SigmaPlot 10.0 software (Jandel Scientific, San Rafael, CA, USA). Data are presented as mean ± SEM. Differences with $P<0.05$ are considered to have statistical significance.

3. Results

3.1. The adaptive A549 cisplatin-resistant cells exhibit malignant ability.

We first analyze the viability of A549 clones adapting to cisplatin as described in the materials and methods section, coupled with parental cells under five M cisplatin for 48h. The results showed that parental A549 cells treated with cisplatin exhibited a mortality ratio of approximately 55-65%, while three A549 clones with two µM-cisplatin adaptation all exhibited higher mortality than parental cells (75-82%) in the presence of the same dose of cisplatin (Fig. 1A), suggesting a sensitizing effect of A549 cells under the intermittent long-period treatment of low-dose cisplatin. Surprisingly, one of the cisplatin adaptation clones named A549cisR exhibited high viability in cisplatin compared to parental cells (80-85% vs 40-55%). The genetic heterogeneity of A549 lung cancer cells might cause these diverse responses after the same cisplatin-pulse-chase treatment.

Next, we more thoroughly investigated the drug resistance of A549cisR cells by treating both A549cisR and parental cells with varying concentrations of cisplatin or paclitaxel for 48 hours, followed by a viability assay. The results demonstrated that cisplatin reduced the viability of parental A549 cells in a dose-dependent manner, with approximately 75% viability at 2.5 µM and 45% viability at 5 µM. Furthermore, taxol administration caused higher mortality of parental A549 cells in a dose-dependent manner (85% viability at 5 nM and 43% viability at 20 nM) (Figs. 1B and 1C). A549cisR cells did not exhibit a significant reduction in viability in treatment with five µM cisplatin and a slight reduction in viability in treatment with 20 nM taxol compared to untreated A549cisR cells. They showed increased viability compared to parental A549 cells (105% vs 43%). These results showed the generation of multiple drug-resistant abilities of A549 cells in the long-term treatment of low-dose cisplatin.

Our previous results suggested different mutually-induced malignant abilities in lung cancer cells [14, 16]; thus, A549cisR cells were subjected to a wound-healing assay in the following experiment. Parental and A549cisR cells were grown until confluence, scratch wounds were created using a pipette tip, and the wound site was photographed over time (0 and 24h). The cell-free area was evaluated, and the cell migration rate was calculated to reduce

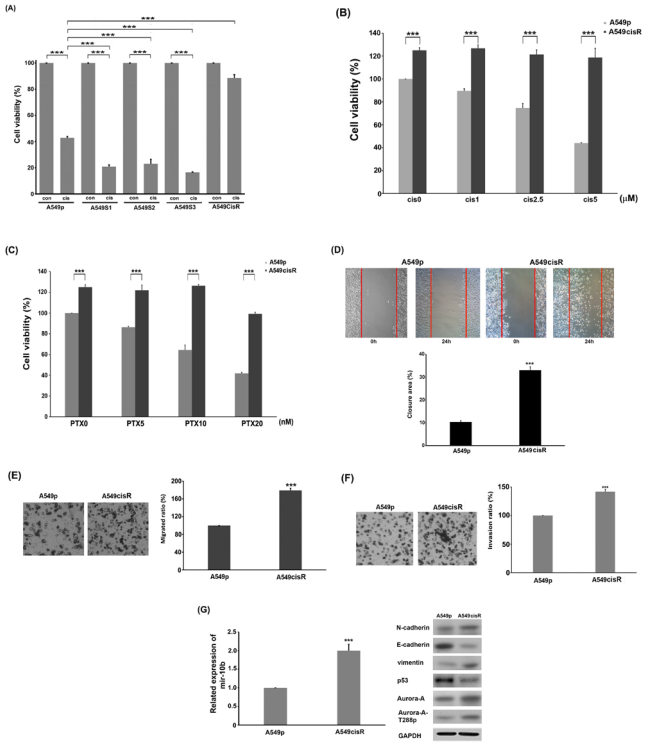

Fig. 1. A549cisR cells showed higher drug resistance and motility than parental cells. (A) The generated clones and parental A549 cells were subjected to cisplatin treatment. ***P<0.001. (B) and (C) The parental or cisplatin-resistant A549 cells were treated with cisplatin (0, 1, 2.5, 5 µM) or paclitaxel (0, 5, 10, 20 nM). 48 h later, the cells were then subjected to MTT assay. The O.D. values of A549cisR cells were then normalized with parental cells as a percentage. (D) The parental or A549cisR cells were subjected to the wound healing assay for 20 h. The closure of the denuded area of A549cisR cells was calculated and then normalized with that of the control. (E) The $2x10^4$ parental or A549cisR cells were subjected to migration assay for 16 h. The migrated cells were then normalized with parental cells. (F) The $1x10^5$ parental or A549cisR cells were subjected to matrigel-coated transwell assay for 24 h. The invasive A549cisR cells were then calculated and normalized with that of parental A549 cells. (G)The parental and A549cisR cells were collected and subjected to protein extraction and western blot to reveal the expression of E-cadherin, N-cadherin, vimentin, p53, and Aurora-A or miRNA extraction followed by qRT-PCR to reveal the expression of mir-10b. * P<0.05, **P<0.005, ***P<0.001.

the cell-free area. Our data demonstrated that A549cisR cells contributed to progressive wound closure. Quantification of the wounded area revealed that there was a larger closure area of A549cisR cells compared to that of parental cells (11% vs. 35%) (Fig. 1D); additionally, A549cisR cells showed a higher migration ability than parental cells (increasing 95% vs. parental cells) (Fig. 1E). Furthermore, parental and A549cisR cells were subjecting to migration and invasion assay. As expected, the results showed that A549CisR cells exhibited a higher migration (increasing by 55% vs. parental cells) and invasion ability (increasing by 35% vs. parental cells) compared to parental cells (Fig. 1F). These results indicated that a progression of motility could also accompany the acquired drug-resistant ability of A549 cells. We previously showed that ectopic expression of mir-10b induces drug-resistant ability and motility simultaneously, which is usually accompanied by the downregulation of p53, which usually exhibits an inversely functional correlation with Aurora-A [20, 23]. In this study, A549cisR cells exhibited multiple malignant characteristics. To uncover possible underlying mechanisms, we analyzed the expressions of molecules related to p53, mir-10b, Aurora-A, and EMT (Epithelial-Mesenchymal Transition). The results showed that n A549cisR cells exhibited a higher expression of mir-10b (increasing by 95% vs. parental cells) and Aurora-A (increasing by 50% vs. parental cells); Aurora-A activity was also elevated by about 70%. Furthermore, the levels of N-cadherin and vimentin increased by 55-80% compared to parental cells. In contrast, the expression of p53 and E-cadherin was reduced by 60%-72% in A549cisR cells (Fig. 1G). These results suggested the possibility of generating multiple malignant tumor cells via intermittent treatment with low doses of cisplatin.

3.2. Suppression of either mir-10b or Aurora-A attenuates malignancy of A549cisR cells

To further characterize the causality between mr-10b and Aurora-A in A549cisR cells, mir-10b antagomirs were transfected into A549cisR cells to knockdown endogenous mir-10b expression, followed by an examination of the levels of p53, Aurora-A, and EMT-related molecules. The results showed that the reduction of mir-10b significantly reduced both Aurora-A protein and activity (reduced by 45 and 65%, respectively). Furthermore, N-cadherin and vimentin levels were down-regulated by 55-75%. In contrast, both p53 and E-cadherin were elevated by 70-90% in mir-10b knockdown A549CisR cells (Fig. 2A). The functional assay showed that the knockdown of mir-10b sensitized A549cisR cells to the treatment of both cisplatin and taxol, the viability of the A549cisR mir-10b knockdown cells was reduced by approximately 40% and 15% under cisplatin or taxol treatment, respectively, in comparison with that of A549cisR cells. (Fig. 2B). Furthermore, A549cisR mir-10b knockdown cells also reduced the invasion ability by 31% in comparison with A549cisR cells (Fig. 2C). These results indicated that elevated mir-10b was responsible for drug resistance and motility in A549cisR cells. Next, we characterized the role of Aurora-A in mir-10b-mediated malignancy of A549cisR cells. Scramble or Aurora-A siRNA was transfected into A549cisR cells, followed by expression and functional assay. The western results showed that Aurora-A knockdown reduced the levels of both vimentin and N-cadherin while inducing those of p53 and E-cadherin (Fig. 2D). Interestingly, Aurora-A knockdown reduced mir-10b level, which could be caused by p53 elevation. As expected, the viability of the A549cisR-Aurora-A siRNA transfectant under treatment with cisplatin or taxol was reduced by approximately 12 and 25%, respectively, compared to that of the A549cisR-scRNA transfectant (Fig. 2E). The invasion ability of the Aurora-A siRNA transfectant A549cisR was also reduced more by approximately 22 % compared to A549cisR cells transfected with scRNA (Fig. 2F).

3.3. Mir-10b and Aurora-A are mutual regulators in priming malignance of A549cisR cells

Next, A549cisR cells were transfected with mir-10b antagomir alone or with Aurora-A-WT-Flag to analyze the correlations between mir-10b and Aurora-A in regulating the malignant ability of lung cancer cells. However, the results were not significant (data not shown). Thus, the Aurora-A-T288D-Flag, a constitutively active form of Aurora-A, was replaced with Aurora-A-WT-Flag in this experiment. The following results showed that the ectopic expression of Aurora-A T-288D recovered the expression of endogenous mir-10b, which was previously reduced in the presence of mir-10b antagomir. Furthermore, both N-

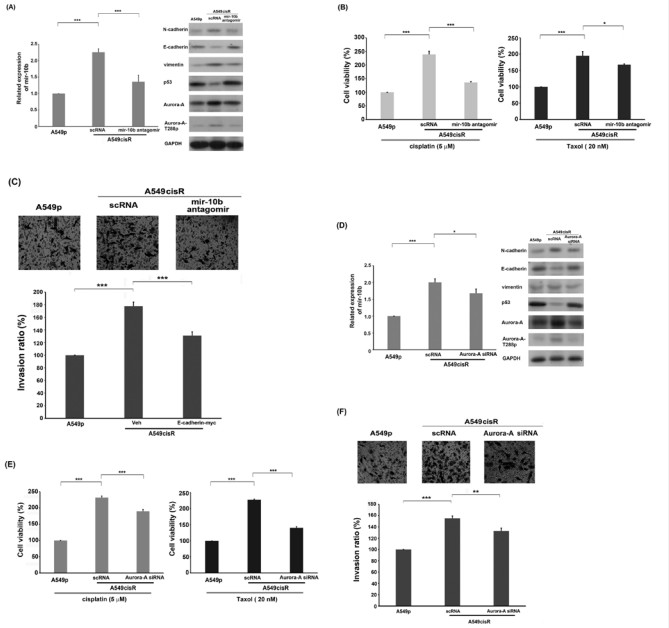

Fig. 2. Suppressing mir-10b or Aurora-A attenuated the malignant properties and the expression levels of EMT-associated proteins in A549cisR cells. (A) The A549cisR cells were transfected with scRNA or mir-10b antagomir, followed by investigating the expressions of EMT-related proteins via western blot procedure, ***P<0.001. (B) and (C) The parental A549 cells, scRNA, or mir-10b antagomir-transfected A549cisR cells were subjected to (B) cisplatin and taxol viability assay or (C) invasion assay, respectively. The results were represented as a related ratio (normalized with that of the parental group). (D) The A549cisR cells were transfected with siRNA or Aurora-A siRNA, followed by investigating the expression of EMT-related proteins via western blot procedure; the expression of mir-10b was also investigated via qPCR procedure. (E) and (F) The parental A549 cells, scRNA, or Aurora-A-siRNA transfected A549cisR cells were subjected to (E) cisplatin or taxol treatment or (F) invasion assay, respectively. The results were represented as a related ratio (normalized with that of the parental group).* P<0.05, **P<0.005, ***P<0.001.

cadherin and vimentin expression levels were recovered. At the same time, p53 and E-cadherin were again down-regulated in mir-10b antagomir and Aurora-A-T288D-Flag contransfected A549cisR cells (Fig. 3A). The reducing viability of mir-10b antagomir transfected A549cisR cells treated with cisplatin or taxol was recovered by 48 % and 71%, respectively, when cotransfected with Aurora-A-T288D flag (Fig. 3B). The motility of mir-10b knockdown A549cisR cells increased by approximately 30% when introduced with Aurora-A-T288D-flag (Fig. 3C). These results suggested that Aurora-A and mir-10b might interact in lung cancer cells. In addition to the gain-of-function assay, the loss-of-function test of mir-10b-Aurora-A was also performed to confirm the causality between mir-10b and Aurora-A. A549cisR cells were transfected with Aurora-A siRNA alone or mir-10b agomir, followed by expression and function analysis. The results showed that the knockdown of Aurora-A reduced the level of mir-10b by 25%, N-cadherin by 50%, and vimentin by 90%, while it increased that of E-cadherin and p53 by approximately 80-90%. Surprisingly, co-expression with mir-10b agomir recovered the expressions of the indicated molecules described above to a greater degree than veh/scRNA transfected A549cisR cells (Fig. 3D). The reducing viability of Aurora-A-knockdown A549cisR cells treating with cisplatin or taxol was recovered in the presence of mir-10b agomir by approximately 30-40%, however, remained lower than veh/scRNA-A549cisR cells (Fig. 3E). On the contrary, the invasion ability of control A549cisR cells attenuated by Aurora-A siRNA was significantly recovered up to that of control A549cisR cells when co-transfected with mir-10b agomir (Fig. 3F). These results suggested that mir-10b and Aurora-A act as a mutual inducer in priming multiple malignant A549 cells under intermittent low-dose cisplatin treatment.

3.4. E-cadherin is an important downstream effector of mir-10b/Aurora-A in regulating malignancy of A549cisR cells

In the following experiment, we characterized the role of E-cadherin in mir-10b priming malignancy of A549cisR cells. The E-cadherin-flag construct was transfected into A549cisR cells. Surprisingly, the Western blot results showed that ectopic expression of E-cadherin reduced the levels of both N-cadherin and vimentin, and Aurora-A activities and expression levels were also attenuated. Furthermore, an elevated mir-10b was reduced. At the same time, a level of p53 was induced in A549cisR cells transfected with E-cadherin (Fig. 4A). Regarding the functional assay, restoration of the level of E-cadherin reduced the viability of A549cisR cells when treated with cisplatin and taxol by approximately 35% and 26%, respectively (Fig. 4B). Furthermore, the motility of A549cisR cells transfected with E-cadherin was also reduced by about 30% (Fig. 4C), suggesting a novel role of E-cadherin in reversing malignant ability by balancing the expressions between mir-10b/Aurora-A and p53 in lung tumor cells.

3.5. A549cisR cells transformed parental cells via increasing secretory mir-10b

It was reported that miRNA serves as a distant regulator in secreted form; therefore, we analyzed whether the secreted level of mir-10b was affected by A549cisR cells. The parental and A549cisR cells medium was collected and subjected to the mir-10b expression assay. The results showed that the mir-10b secreted was 55% higher in A549cisR cells (Fig. 5A). The result implied the possibility that cancer cells might assimilate distant cells to transform or maintain malignancy by secreting oncogenic mir-10b. In order to confirm this hypothesis, a conditio-

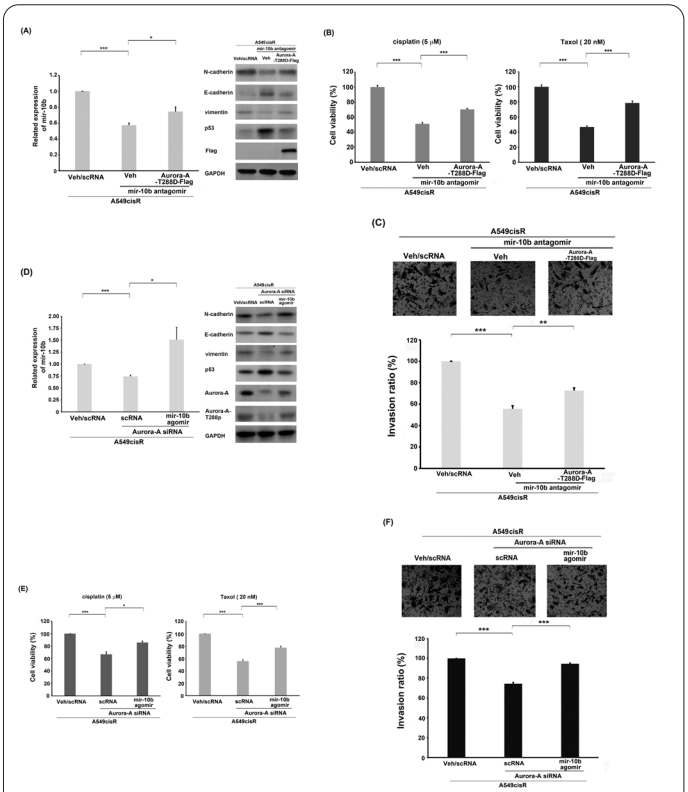

Fig. 3. The Aurora-A and mir-10b acted as mutual effectors in drug resistance and motility in A549cisR cells. (A) The mir-10b-knockdown A549cisR cells were transfected with vehicle or Aurora-A-T288D construct, followed by investigating the expressions of mir-10b or EMT-related proteins. Besides, the (B) drug-resistant and (C) invasion abilities were also investigated, respectively. ***P<0.001. (D) The Aurora-A-knockdown A549cisR cells were transfected with scRNA or mir-10b agomir, followed by investigating the expressions of mir-10b or EMT-related proteins or (E) drug-resistant ability and (F) invasion ability, respectively. * P<0.05, **P<0.005, ***P<0.001.

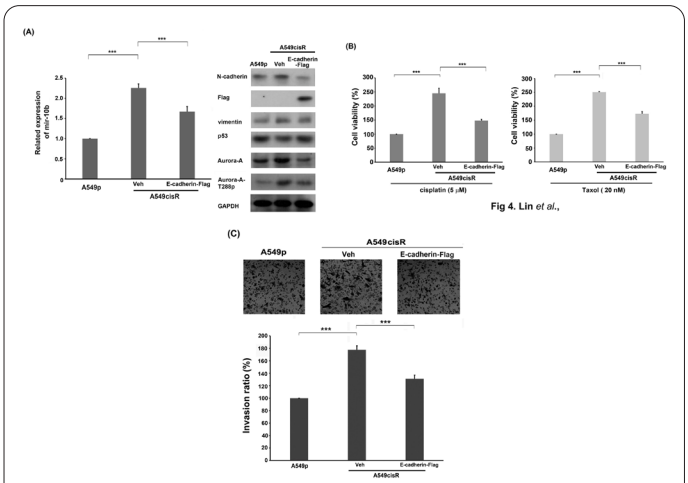

Fig. 4. Overexpression of E-cadherin attenuated drug resistance and motility of A549cisR cells. The A549cisR cells were transfected with E-cadherin-flag, followed by investigating the (A) expressions of mir-10b or EMT-related proteins or (B) drug-resistant ability and (C) invasion ability, respectively, ***P<0.001.

ned medium of A549cisR cells was added to A549 cells for 1 week, and subsequent effects were investigated. The results showed that the levels of mir-10b, N-cadherin, Aurora-A, and Aurora-A-T288p of A549 cells were upregulated by approximately 35-85%, while the levels of E-cadherin and p53 were reduced by 45-65% when engaged with the conditioned medium of A549cisR cells (Fig. 5B). On the contrary, the conditioned medium of A549cisR cells coupled with mir-10b antagomir significantly attenuated the upregulation of N-cadherin, vimentin while downregulation of E-cadherin and p53 expression levels in A549 cells. Furthermore, both Aurora-A activity and expression of A549 cells maintained in A549cisR conditioned medium with mir-10b antagomir remained a similar pattern compared to that of A549cisR-scRNA conditioned medium (Fig. 5B). The viability of A549 cells cultured with conditioned medium to the treatment of cisplatin and taxol increased by approximately 90-105%; while reduced by 30-50 % when cultured with conditioned medium containing mir-10b antagomir (Fig. 5C). A similar result was observed in the motility assay, the invasion ability of A549 cells increased significantly by 90% when cultured with A549cisR-conditioned medium, however, attenuated by 70-80% when A549cisR-conditioned medium containing mir-10b antagomir (Fig. 5D). These results strongly suggested that elevated mir-10b in A549 cells after long-term intermitter treatment with low-dose cisplatin might be secreted by cells and communicate to cells at long distances to the main oncogenic effects.

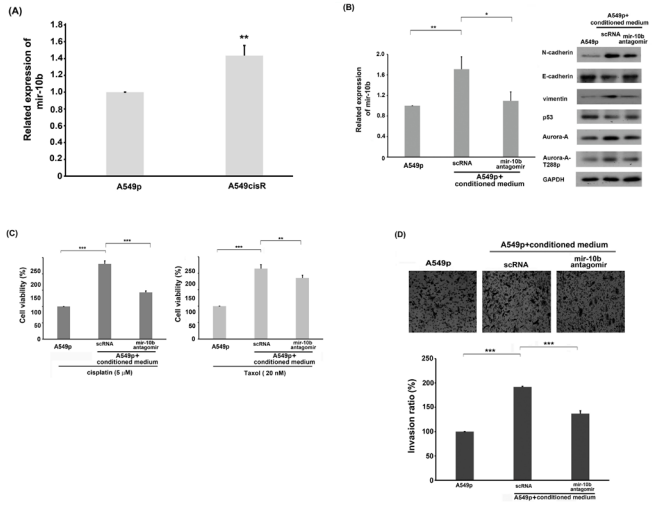

Fig. 5. The secreted mir-10b secreted by A549cisR cells increased the malignant ability and levels of N-cadherin, vimentin, Aurora-A, and mir-10b while reducing that of p53 and E-cadherin in A549 parental cells. (A) The medium of the parental or A549cisR cells was collected and subjected to the mir-10b expression assay procedure. ***P<0.001. (B) The parental A549 cells were incubated with a conditioned medium of A549cisR cells alone or with mir-10b antagomir. One week later, the parental or conditioned-medium cultured A549 cells with or without mir-10b antagomir were subjected to investigate the expressions of mir-10b or EMT-related proteins or (C) drug-resistant and (D) invasion ability, respectively. *P<0.05, **P<0.005

4. Discussion

Currently, cisplatin-based chemotherapy continues to be a primary treatment for patients with NSCLC. However, the development of acquired drug resistance and increased motility often occurs with this therapy. Identifying the mechanisms underlying chemoresistance in NSCLC in current cancer treatments is crucial. Research has shown that miRNA disarray is related to drug resistance in cancers, and addressing miRNA deregulation offers a promising approach to counteracting acquired resistance in NSCLC. In this study, we periodically exposed A549 cells to cisplatin (treated once for two days and rested for another two days) stepwise from 0.5 to 5 μM by increasing 0.5 μM every two weeks to analyze whether A549 cells were eventually able to adapt to high doses of cisplatin. However, A549 cells became unstable and eventually senescent when the cisplatin concentration exceeded two μM (data not shown). Thus, the descendant of 1.5 μM cisplatin-treated A549 cells was applied in this study. As shown in Figure 1, most of the decedent cells showed sensations for high-dose cisplatin treatment (5 μM). Our approach was similar to metronomic chemotherapy except for high-dose, continuous, and longer-duration treatment [17], and is different from that which leads to the gain of cisplatin-resistant cells by high-dose cisplatin treatment in a related short-duration. However, it was not known whether metronomic chemotherapy sensitized A549 cells to cisplatin treatment; our results strongly suggested that pretreatment with low doses of cisplatin could enhance A549 cell mortality when subsequently treated with a higher dose of cisplatin. However, one clone of these A549 cisplatin-training descendants showed resistance to high doses of cisplatin (Fig. 1), similar to the consequence of the metronomic treatment of lung cancer cells [18]. The result might be caused by the intrinsic heterogeneity of A549 cells, which also suggests a possibility of increased drug-resistant capacity after long-term treatment with low-dose chemotherapeutic drug.

Our findings showed that A549cisR cells were also resistant to another chemotherapeutic drug, paclitaxel (Fig. 1), which was in agreement with previous reports that mentioned the generation of cross-resistance to multiple chemotherapeutic drugs, including paclitaxel in cisplatin-resistant cancer cells such as ovarian, leukemia, hepatocellular carcinoma, bladder and lung cancer, etc.[12, 24-27]. Some molecules were involved in parallel resistance of lung cancer cells, such as multidrug resistance (MDR), p-glycoprotein, NF-κB, and p53 [28-32]. Our results showed that the p53 expression was reduced in A549cisR cells; on the contrary, both the activity and expression of Aurora-A, a mutual inhibitor of p53, were upregulated (Fig. 1). Previous reports indicated that Aurora-A participated in the resistance of anti-microtubule drugs such as paclitaxel in cancer cells [33]. This might be one of the reasons that cross-resistance to paclitaxel was raised in cisplatin-resistant lung cancer cells.

Mir-10b was initially identified as a motility promoter of various cancer cells and exhibited high expression in malignant tumors [34]. Recently, our and other studies showed mir-10b is involved in drug resistance in various cancer cells such as lung, breast, esophageal, and hepatocellular carcinoma [23, 35-37]. Wu et al. identified PPAR as a target of mir-10b in cisplatin resistance in esophageal cancer cells[38], and Shao et al. confirmed that cyclin E is targeted by mir-10b against sorafenib toxicity in hepatocellular carcinoma [37]. Ahmad et al. reported that HDAC4 is involved in mir-10b-mediated tamoxifen resistance in breast cancer cells[36]. Our study showed that overexpres-

sion of mir-10b promoted cisplatin resistance by reducing p53 expression and binding it to the 3'UTR region of p53 mRNA [23]. p53 is critical in regulating cisplatin response in cancer cells, and the status of p53 usually determines the therapeutic effects of cisplatin on cancer cells [39]. In this study, the expression of p53 in A549cisR cells was lower than in parental cells (Fig. 4), which might contribute to both cisplatin and taxol resistance. Besides, the E-cadherin, a direct target of mir-10b [40], was downregulated, while the N-cadherin and vimentin were upregulated in A549cisR cells (Fig. 4). E-cadherin is a marker of epithelial cells, and its reduction, accompanied by the increase of N-cadherin and vimentin, is recognized as a malignant transition of tumor cells [41, 42]. It was previously reported that inhibition of p53 repressed the expression of E-cadherin via promoter methylation [43]. In this study, we proposed an alternative pathway underlying E-cadherin reduction mediated by direct inhibition by the increasing mir-10b (Figs. 4 and 5).

Aurora-A is an oncogenic kinase that regulates drug resistance and invasion ability [19, 44]. We previously demonstrated that Aurora-A acted as a mutual repressor with p53 [20]. In the following experiments, we found that mir-10b exhibited a similar pattern to that of Aurora-A in cancer patients (data not shown); besides, in present study, both mir-10b and Aurora-A were upregulated in A549cisR cells (Fig. 1), and mir-10b and Aurora-A might be a mutual activator, since the knockdown of mir-10b reduced both level and activity of Aurora-A while induced that of p53 in A549cisR cells and vice versa (Figs. 2-3). Besides, the re-expression of mir-10b agomir restored the resistance ability against both cisplatin and taxol and the invasion ability attenuated by Aurora-A-siRNA in A549cisR cells (Fig. 3). It was reported that Aurora-A induces an aggressive phenotype via phosphorylating Twist-1 in pancreatic carcinoma [45]. In addition, Twist-1 acts as a transcriptional activator of mir-10b [46]; these results might explain the positive regulation of Aurora-A on mir-10b in A549cisR cells. Interestingly, the overexpression of E-cadherin attenuated the expressions of mir-10b and Aurora-A while induced that of p53 accompanied with the restoration of sensitivity to cisplatin and taxol; besides, the aggressive motility of A549cisR cells was also reduced when transfected with E-cadherin construct (Fig. 4). Song *et al*., summarized that E-cadherin induced by natural compounds such as berberin involved in the attenuation of drug-resistance[47]. Sasaki *et al*. mentioned the recovery of E-cadherin sensitized chemotherapeutic drug by negatively regulating the expression of Bcl-2 [48]. Our results indicated that E-cadherin might also regulate other important molecules, such as mir-10b and Aurora-A, in transforming tumor cells.

It was reported that exosome-mediated mir-10b promotes the malignant transformation of breast tumor cells [49]; besides, circulated mir-10b is recognized as a predictor of non-small cell lung cancer progression [50]. In our study, we first demonstrated that the A549cisR cells produced extracellular mir-10b, which increased the malignant ability of A549 cells, plus with the induction of mir-10b, Aurora-A, N-cadherin and vimentin while reducing p53(Fig. 5), suggesting a spread effect of secretory mir-10b in triggering malignant ability of A549 cells.

Conclusively, these findings raised an interpretation that long-period intermittent treatment of cisplatin sensitized most A549 cells; however, small amounts of intrinsic-resistant cells raised and gained anti-chemotherapeutic drugs and aggressive motility via mir-10-p53/Aurora-A-E-cadherin pathways.

In summary, our study reveals a crucial regulatory network involving mir-10b, Aurora-A, p53, and E-cadherin that orchestrates cisplatin resistance and invasive characteristics in lung cancer cells. We demonstrated that intermittent low-dose cisplatin treatment induces a subpopulation of A549 cells (A549cisR) with elevated mir-10b and Aurora-A levels, accompanied by suppressed p53 and E-cadherin expression. This molecular interplay enhances both drug resistance and malignant motility. Importantly, mir-10b and Aurora-A mutually reinforce each other's expression and function, driving the progression of malignancy. Restoration of E-cadherin expression significantly attenuates these aggressive phenotypes, highlighting its pivotal downstream role. Moreover, secreted mir-10b from resistant cells promotes malignant transformation in parental cells, suggesting a paracrine mechanism contributing to tumor heterogeneity and progression. These findings provide novel insights into the molecular mechanisms underlying chemoresistance and metastasis in non-small cell lung cancer and propose the mir-10b/Aurora-A/p53/E-cadherin axis as a promising therapeutic target to overcome drug resistance and tumor invasiveness.

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